Practitioner's Docket No. 81839

CHAPTER II

Preliminary Classification:
Proposed Class:
Subclass:

NOTE: *"All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.'" M.P.E.P., § 601, 7th ed.*

TRANSMITTAL LETTER TO THE UNITED STATES ELECTED OFFICE (EO/US)

(ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II)

PCT/DE00/02924	28 AUGUST 2000	31 AUGUST 1999
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED

METHODS FOR THE PRODUCTION OF A CHANNEL-FORMING PROTEIN
TITLE OF INVENTION

MICHAEL NIEDERWEIS and STEFAN BOSSMANN
APPLICANT(S)

Box PCT
Assistant Commissioner for Patents
Washington D.C. 20231

ATTENTION: EO/US

CERTIFICATION UNDER 37 C.F.R. § 1.10*
(Express Mail label number is mandatory.)
(Express Mail certification is optional.)

I hereby certify that this Transmittal Letter and the papers indicated as being transmitted therewith is being deposited with the United States Postal Service on this date Feb. 28, 2002, in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EL919995927US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

EDWARD M. KRIEGSMAN
(type or print name of person mailing paper)

Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

WARNING: *Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).*
*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.*

NOTE: To avoid abandonment of the application, the applicant shall furnish to the USPTO, not later than 20 months from the priority date. (1) a copy of the international application, unless it has been previously communicated by the International Bureau or unless it was originally filed in the USPTO; and (2) the basic national fee (see 37 C.F.R. § 1.492(a)). The 30-month time limit may not be extended 37 C.F.R. § 1.495.

***WARNING:** Where the items are those which can be submitted to complete the entry of the international application into the national phase are subsequent to 30 months from the priority date the application is still considered to be in the international state and if mailing procedures are utilized to obtain a date the express mail procedure of 37 C.F.R. § 1.10 must be used (since international application papers are not covered by an ordinary certificate of mailing—See 37 C.F.R. § 1.8.*

NOTE: Documents and fees must be clearly identified as a submission to enter the national state under 35 U.S.C. § 371 otherwise the submission will be considered as being made under 35 U.S.C. § 111. 37 C.F.R. § 1.494(f).

I. Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. § 371:

a. ☒ This express request to immediately begin national examination procedures (35 U.S.C. § 371(f)).

b. ☒ The U.S. National Fee (35 U.S.C. § 371(c)(1)) and other fees (37 C.F.R. § 1.492) as indicated below:

2. Fees

CLAIMS FEE	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
☒*	TOTAL CLAIMS	40 -20=	20	× $18.00=	$ 360
	INDEPENDENT CLAIMS	11 -3=	8	$84 × ~~$78.00~~=	672
	MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $260.00	0
BASIC FEE**	☐ U.S. PTO WAS INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where an international preliminary examination fee as set forth in § 1.482 has been paid on the international application to the U.S. PTO: ☐ and the international preliminary examination report states that the criteria of novelty, inventive step (non-obviousness) and industrial activity, as defined in PCT Article 33(1) to (4) have been satisfied for all the claims presented in the application entering the national stage (37 C.F.R. § 1.492(a)(4)) ..... $96.00 ☐ and the above requirements are not met (37 C.F.R. § 1.492(a)(1)) ..... $670.00 ☒ U.S. PTO WAS NOT INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where no international preliminary examination fee as set forth in § 1.482 has been paid to the U.S. PTO, and payment of an international search fee as set forth in § 1.445(a)(2) to the U.S. PTO: ☐ has been paid (37 C.F.R. § 1.492(a)(2)) ..... $690.00 ☐ has not been paid (37 C.F.R. § 1.492(a)(3)) ..... $970.00 ☒ where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 C.F.R. § 1.492(a)(5)) ..... ~~$840.00~~ 890				890
				Total of above Calculations	=1922
SMALL ENTITY	Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (note 37 C.F.R. § 1.9, 1.27, 1.28)				- 961
				Subtotal	961
				Total National Fee	$ 961
	Fee for recording the enclosed assignment document $40.00 (37 C.F.R. § 1.21(h)). (See Item 13 below). See attached "ASSIGNMENT COVER SHEET".				0
TOTAL				Total Fees enclosed	$ 961

>PLICANT IS
SMALL ENTIT

*See attached Preliminary Amendment Reducing the Number of Claims.

i. ☒ A check in the amount of 961 to cover the above fees is enclosed.

ii. ☐ Please charge Account No. _____ in the amount of $ _____. A duplicate copy of this sheet is enclosed.

**WARNING: *"To avoid abandonment of the application the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 30 months from the priority date: * * * (2) the basic national fee (see § 1.492(a)). The 30-month time limit may not be extended." 37 C.F.R. § 1.495(b).*

WARNING: *If the translation of the international application and/or the oath or declaration have not been submitted by the applicant within thirty (30) months from the priority date, such requirements may be met within a time period set by the Office. 37 C.F.R. § 1.495(b)(2). The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than thirty (30) months after the priority date. The payment of the processing fee set forth in § 1.492(f) is required for acceptance of an English translation later than thirty (30) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1.136 apply to the period which is set. Notice of Jan. 3, 1993, 1147 O.G. 29 to 40.*

3. ☒ A copy of the International application as filed (35 U.S.C. § 371(c)(2)):

NOTE: *Section 1.495 (b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 30 months from the priority date to avoid abandonment. "The International Bureau normally provides the copy of the international application to the Office in accordance with PCT Article 20. At the same time, the International Bureau notifies applicant of the communication to the Office. In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage, the applicant normally need only check to be sure the notice from the International Bureau has been received and then pay the basic national fee by 30 months from the priority date." Notice of Jan. 7, 1993, 1147 O.G. 29 to 40, at 35-36. See item 14c below.*

a. ☒ is transmitted herewith.

b. ☐ is not required, as the application was filed with the United States Receiving Office.

c. ☐ has been transmitted

i. ☐ by the International Bureau.
Date of mailing of the application (from form PCT/1B/308): _____.

ii. ☐ by applicant on _____.
Date

4. ☒ A translation of the International application into the English language (35 U.S.C. § 371(c)(2)):

a. ☒ is transmitted herewith.

b. ☐ is not required as the application was filed in English.

c. ☐ was previously transmitted by applicant on _____.
Date

d. ☐ will follow.

5. ☐ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. § 371(c)(3)):

NOTE: *The Notice of January 7, 1993 points out that 37 C.F.R. § 1.495(a) was amended to clarify the existing and continuing practice that PCT Article 19 amendments must be submitted by 30 months from the priority date and this deadline may not be extended. The Notice further advises that: "The failure to do so will not result in loss of the subject matter of the PCT Article 19 amendments. Applicant may submit that subject matter in a preliminary amendment filed under section 1.121. In many cases, filing an amendment under section 1.121 is preferable since grammatical or idiomatic errors may be corrected." 1147 O.G. 29-40, at 36.*

a. ☐ are transmitted herewith.

b. ☐ have been transmitted

i. ☐ by the International Bureau.
Date of mailing of the amendment (from form PCT/1B/308): _____

ii. ☐ by applicant on (date) _____
Date

c. ☐ have not been transmitted as

i. ☐ applicant chose not to make amendments under PCT Article 19.
Date of mailing of Search Report (from form PCT/ISA/210.): _____.

ii. ☐ the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

6. ☐ A translation of the amendments to the claims under PCT Article 19 (38 U.S.C. § 371(c)(3)):

a. ☐ is transmitted herewith.

b. ☐ is not required as the amendments were made in the English language.

c. ☐ has not been transmitted for reasons indicated at point 5(c) above.

7. ☐ A copy of the international examination report (PCT/IPEA/409)

☐ is transmitted herewith.

☐ is not required as the application was filed with the United States Receiving Office.

8. ☐ Annex(es) to the international preliminary examination report

a. ☐ is/are transmitted herewith.

b. ☐ is/are not required as the application was filed with the United States Receiving Office.

9. ☐ A translation of the annexes to the international preliminary examination report

a. ☐ is transmitted herewith.

b. ☐ is not required as the annexes are in the English language.

10. ☒ An oath or declaration of the inventor (35 U.S.C. § 371(c)(4)) complying with 35 U.S.C. § 115

a. ☐ was previously submitted by applicant on _____.
Date

b. ☒ is submitted herewith, and such oath or declaration

i. ☒ is attached to the application.

ii. ☐ identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or 3(c) and 5(b); and states that they were reviewed by the inventor as required by 37 C.F.R. § 1.70.

c. ☐ will follow.

II. Other document(s) or information included:

11. ☐ An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a):

a. ☐ is transmitted herewith.

b. ☐ has been transmitted by the International Bureau.
Date of mailing (from form PCT/IB/308): _____.

c. ☐ is not required, as the application was searched by the United States International Searching Authority.

d. ☐ will be transmitted promptly upon request.

e. ☐ has been submitted by applicant on _____.
Date

12. ☒ An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98:

a. ☒ is transmitted herewith.

Also transmitted herewith is/are:

☐ Form PTO-1449 (PTO/SB/08A and 08B).

☒ Copies of citations listed.

b. ☐ will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. § 371(c).

c. ☐ was previously submitted by applicant on _____.
Date

13. ☐ An assignment document is transmitted herewith for recording.

A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO 1595 is also attached.

_____

_____

_____

14. ☒ Additional documents:
    a. ☐ Copy of request (PCT/RO/101)
    b. ☐ International Publication No. _____
        i. ☐ Specification, claims and drawing
        ii. ☐ Front page only
    c. ☒ Preliminary amendment (37 C.F.R. § 1.121)
    d. ☐ Other

15. ☒ The above checked items are being transmitted
    a. ☒ before 30 months from any claimed priority date.
    b. ☐ after 30 months.

16. ☐ Certain requirements under 35 U.S.C. § 371 were previously submitted by the applicant on _____, namely:

AUTHORIZATION TO CHARGE ADDITIONAL FEES

WARNING: *Accurately count claims, especially multiple dependant claims, to avoid unexpected high charges if extra claims are authorized.*

NOTE: "A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).

NOTE: "Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

☒ The Commissioner is hereby authorized to charge the following additional fees that may be required by this paper and during the entire pendency of this application to Account No. 11-1755

☒ 37 C.F.R. § 1.492(a)(1), (2), (3), and (4) (filing fees)

WARNING: *Because failure to pay the national fee within 30 months without extension (37 C.F.R. § 1.495(b)(2)) results in abandonment of the application, it would be best to always check the above box.*

☐ 37 C.F.R. § 1.492(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.492(d)), it might be best not to authorize the PTO to charge additional claim fees, except possible when dealing with amendments after final action.

☐ 37 C.F.R. § 1.17 (application processing fees)

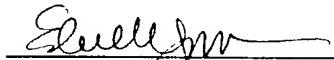
☐ 37 C.F.R. § 1.17(a)(1)–(5) (extension fees pursuant to § 1.136(a).

☐ 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

NOTE: 37 C.F.R. § 1.28(b) requires "Notification of any change in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying . . . issue fee." From the wording of 37 C.F.R. § 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

☐ 37 C.F.R. § 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 30 months after the priority date).



SIGNATURE OF PRACTITIONER

Reg. No.: 33,529

EDWARD M. KRIEGSMAN
(type or print name of practitioner)

Tel. No.: (508) 879-3500

KRIEGSMAN & KRIEGSMAN
665 FRANKLIN STREET
P.O. Address

Customer No.: 23685

FRAMINGHAM, MA 01702

PATENT
Attorney Docket No. 81839

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

MICHAEL NIEDERWEIS ET AL.

Serial No.: 10/070,099 — Group Art Unit: Unknown

Int'l. Appl. Filing Date: August 28, 2000 — Examiner: Unknown

For: METHODS FOR THE PRODUCTION OF A CHANNEL-FORMING PROTEIN

U.S. Patent and Trademark Office
Box Sequence, P.O. Box 2327
Arlington, VA 22202

Sir:

TRANSMITTAL OF COMPUTER READABLE FORM OF SEQUENCE LISTING

In connection with the above-identified patent application, Applicants are transmitting herewith a computer readable copy of the Sequence Listing filed with the present application.

The Sequence Listing information recorded in computer readable form is identical to the written on paper sequence listing. No new matter is added thereby.

If there are any fees due in connection with the filing of this paper that are not accounted for, the Examiner is authorized to charge the fees to our Deposit Account No. 11-1755. If a fee is

PATENT
Attorney Docket No. 81839

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
MICHAEL NIEDERWEIS ET AL.	)	
	)	
Serial No.: Unassigned	)	Group Art Unit: Unknown
	)	
Filed: Herewith	)	Examiner: Unknown
	)	
For: METHODS FOR THE PRODUCTION OF A CHANNEL-FORMING PROTEIN	)	

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT

Prior to examination of the above-identified patent application, please enter the amendment indicated below.

IN THE CLAIMS:

Please cancel claim 33 without prejudice or disclaimer of the subject matter thereof and please amend claims 1-5, 7-26, 32, 34-35 and 37-39 as follows:

1. (Amended) A Method for producing a channel-forming protein, found in gram-positive bacteria, wherein the channel-forming protein is obtained by

a) heterologous overexpression or

b) purification from mycobacteria, wherein the extraction temperature is higher than 50°C.

2. (Amended) A Method according to claim 1, wherein the gram-positive bacterium is one that contains at least one mycolic acid.

3. (Amended) A Method according to claim 2, wherein the bacterium is a mycobacterium, preferably *Mycobacterium smegmatis*.

4. (Amended) A Method according to claim 1, wherein the channel forming protein is a porin.

5. (Amended) A Method according to claim 4, wherein the porin is essentially chemically stable against organic solvents.

7. (Amended) A Method according to claim 1, wherein the porin is the porin MspA, MspC, MspD, a fragment of one of these porins, a homologous protein from one of these porins or their fragments, or a protein taken from a sequence of one of these porins.

8. (Amended) A Method according to claim 1, wherein the heterologous overexpression is realized in *E. coli* or mycobacteria.

9. (Amended) A Method according to claim 1, wherein a gene encoding a channel-forming protein, preferably a porin, is overexpressed.

10. (Amended) A Method according to claim 1, wherein an *mspA* gene according to sequence 1, an *mspC* gene according to sequence 6, or an *mspD* gene according to sequence 8 is overexpressed.

11. (Amended) A Method according to claim 10, wherein a mutant gene derived from the sequences 1, 6, or 8 is overexpressed, in which the mutation is essentially so that the chemical and thermal stability, as well as the channel-like structure, correspond essentially with that of MspA, MspC or MspD.

12. (Amended) A Method according to claim 11, wherein the mutation is essentially so that the codon usage of the *mspA*, *mspC* or *mspD* gene is adapted to that of highly expressed genes in *E. coli*.

13. (Amended) A Method according to claim 11, wherein a mutated *mspA*-, *mspC*- or *mspD* gene is used for overexpression where the mutation is essentially so that the G+C content is reduced to less than 66%.

14. (Amended) A Method according to claim 1, wherein the *synmspA* gene according to sequence 4 is overexpressed.

15. (Amended) A Method according to claim 14, wherein a suitable vector, containing the *synmspA* gene according to sequence 4, is used for overexpression in *E. coli*.

16. (Amended) A Method according to claim 1, wherein the channel-forming proteins are produced from the cell wall from gram-positive bacteria using non-ionic or zwitterionic detergents.

17. (Amended) A Method according to claim 16, wherein the detergents used come from the following list: isotridecylpoly(ethyleneglycolether)$_n$, alkylglucosides, especially octylglucoside, alkylmaltoside, especially dodecylmaltoside, alkylthioglucosides, especially octylthioglucoside, octyl-polyethylenoxide and lauryldimethylaminoxide.

18. (Amended) A Method according to claim 1, wherein the extraction temperature is between 80 and 110°C, preferably between 90 and 100°C.

19. (Amended) A Method according to claim 1, wherein the extraction time is 5-120 min, preferably 25-35 min.

20. (Amended) A Method according to claim 1, wherein a buffer with an ionic strength above 50 mM NaCl or Na-phosphate is used.

21. (Amended) A Method according to claim 1, wherein the channel-forming protein is purified by precipitation, particularly using acetone.

22. (Amended) A Method according to claim 1, wherein the channel-forming protein is purified using ion-exchange chromatography, particularly an anion-exchange chromatography.

23. (Amended) A Method according to claim 1, wherein the channel-forming protein is purified using size-exclusion chromatography.

24. (Amended) A Method according to claim 1, wherein the channel-forming protein, produced through heterologous overexpression by raising its local concentration, is renatured.

25. (Amended) A Method according to claim 24, wherein raising of the local protein concentration is realized by electrophoretic enrichment, especially by means of a DC current, by precipitation or adsorption at a suitable surface, especially at a membrane.

26. (Amended) Channel-forming protein from a gram-positive bacterium, produced according to a method according to claim 1.

32. (Amended) Gene, wherein the gene is the *mspA* gene according to sequence 1.

34. (Amended) Gene, wherein the gene is the *mspC* gene according to sequence 6.

35. (Amended) Gene, wherein the gene is the *mspD* gene according to sequence 8.

37. (Amended) Mutated *mspA* gene, *mspC* gene or *mspD* gene, according to claim 36, in which the mutation essentially consists of reducing the G+C content to less than 66%.

38. (Amended) Mutated *mspA* gene, *mspC* gene or *mspD* gene, according to claim 36, derived from one of the sequences 1, 6, or 8, in which the mutation is such that the chemical and thermal stability, as well as the channel-like structure of the protein is for all practical purposes that of MspA, MspC or MspD.

39. (Amended) Mutated *mspA* gene, wherein the mutated gene is the *synmspA* gene according to sequence 4.

REMARKS

Claim 33 has been canceled herein. Claims 1-5, 7-26, 32, 34-35 and 37-39 have been amended herein. No new claims have been added herein. Therefore, claims 1-32 and 34-41 are under active consideration.

If there are any fees due in connection with the filing of this paper that are not accounted for, the Examiner is authorized to charge the fees to our Deposit Account No. 11-1755. If a fee is required for an extension of time under 37 C.F.R. 1.136 that is not accounted for already, such an extension of time is requested and the fee should also be charged to our Deposit Account.

Respectfully submitted,

Kriegsman & Kriegsman

By: _____

Edward M. Kriegsman
Reg. No. 33,529
665 Franklin Street
Framingham, MA 01702
(508) 879-3500

Dated: February 28, 2002

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231 on _____.

_____
Edward M. Kriegsman
Reg. No. 33,529
Dated:_____

MARKED-UP AMENDED CLAIMS 1-5, 7-26, 32, 34-35 and 37-39

1. (Amended) A Method for producing a channel-forming protein, found in gram-positive bacteria, wherein the channel-forming protein is obtained by

a) heterologous overexpression or

b) purification from mycobacteria, [whereby] wherein the extraction temperature is higher than 50°C.

2. (Amended) A Method according to [one of the aforementioned claims] claim 1, wherein the gram-positive bacterium is one that contains at least one mycolic acid.

3. (Amended) A Method according to [one of the aforementioned claims] claim 2, wherein the bacterium is a mycobacterium, preferably *Mycobacterium smegmatis*.

4. (Amended) A Method according to [one of the aforementioned claims] claim 1, wherein the channel forming protein is a porin.

5. (Amended) A Method according to [one of the aforementioned claims] claim 4, wherein the porin is essentially chemically stable against organic solvents.

7. (Amended) A Method according to [one of the aforementioned claims] claim 1, wherein the porin is the porin MspA, MspC, MspD, a fragment of one of these porins, a homologous protein from one of these porins or their fragments, or a protein taken from a sequence of one of these porins.

8. (Amended) A Method according to [one of the aforementioned claims] claim 1, wherein the heterologous overexpression is realized in *E. coli* or mycobacteria.

9. (Amended) A Method according to [one of the aforementioned claims] claim 1, wherein a gene encoding a channel-forming protein, preferably a porin, is [used for the overexpression] overexpressed.

10. (Amended) A Method according to [one of the aforementioned claims] claim 1, wherein an *mspA* gene according to sequence 1, an *mspC* gene according to sequence 6, or an *mspD* gene according to sequence 8 is [used for overexpression] overexpressed.

11. (Amended) A Method according to [one of the aforementioned claims] claim 10, wherein a mutant gene derived from the sequences 1, 6, or 8 is [used for overexpression] overexpressed, in which the mutation is essentially so that the chemical and thermal stability, as well as the channel-like structure, correspond essentially with that of MspA, MspC or MspD.

12. (Amended) A Method according to [one of the aforementioned claims] claim 11, wherein the mutation is essentially so that the codon usage of the *mspA*, *mspC* or *mspD* gene is adapted to that of highly expressed genes in *E. coli*.

13. (Amended) A Method according to [one of the aforementioned claims] claim 11, wherein a mutated *mspA*-, *mspC*- or *mspD* gene is used for overexpression where the mutation is essentially so that the G+C content is reduced to less than 66%.

14. (Amended) A Method according to [one of the aforementioned claims] claim 1, wherein the *synmspA* gene according to sequence 4[,] is [used for overexpression] overexpressed.

15. (Amended) A Method according to [one of the aforementioned claims] claim 14, wherein a suitable vector [for overexpression in *E. coli*], containing the *synmspA* gene according to sequence 4, is used for overexpression in *E. coli*.

[redacted]

16. (Amended) A Method according to [one of the aforementioned claims] claim 1, wherein the channel-forming proteins are produced from the cell wall from gram-positive bacteria using non-ionic or zwitterionic detergents.

17. (Amended) A Method according to [one of the aforementioned claims] claim 16, wherein the detergents used come from the following list: isotridecylpoly(ethyleneglycolether)$_n$, alkylglucosides, especially octylglucoside, alkylmaltoside, especially dodecylmaltoside, alkylthioglucosides, especially octylthioglucoside, octyl-polyethylenoxide and lauryldimethylaminoxide.

18. (Amended) A Method according to [one of the aforementioned claims] claim 1, wherein the extraction temperature is between 80 and 110°C, preferably between 90 and 100°C.

19. (Amended) A Method according to [one of the aforementioned claims] claim 1, wherein the extraction time is 5-120 min, preferably 25-35 min.

20. (Amended) A Method according to [one of the aforementioned claims] claim 1, wherein a buffer with an ionic strength above 50 mM NaCl or Na-phosphate is used.

21. (Amended) A Method according to [one of the aforementioned claims] claim 1, wherein the channel-forming protein is purified by precipitation, particularly using acetone.

22. (Amended) A Method according to [one of the aforementioned claims] claim 1, wherein the channel-forming protein is purified using ion-exchange chromatography, particularly an anion-exchange chromatography.

23. (Amended) A Method according to [one of the aforementioned claims] claim 1, wherein the channel-forming protein is purified using size-exclusion chromatography.

24. (Amended) A Method according to [one of the aforementioned claims] claim 1, [wherein one of the aforementioned claims,] wherein the channel-forming protein, produced through heterologous overexpression by raising [the] its local concentration, is renatured.

25. (Amended) A Method according to claim 24, wherein raising of the local protein concentration is realized by electrophoretic enrichment, especially by means of a DC current, by precipitation or adsorption at a suitable surface, especially at a membrane.

26. (Amended) Channel-forming protein from a gram-positive bacterium, produced according to a method according to [one of the aforementioned claims] claim 1.

32. (Amended) Gene, [encoding a channel forming protein according to one of the claims 26-31] wherein the gene is the *mspA* gene according to sequence 1.

34. (Amended) Gene [according to claim 32], wherein the gene is the *mspC* gene according to sequence 6.

35. (Amended) Gene [according to claim 32], wherein the gene is the *mspD* gene according to sequence 8.

37. (Amended) Mutated *mspA* gene, *mspC* gene or *mspD* gene, [in particular] according to claim 36, in which the mutation essentially consists of reducing the G+C content to less than 66%.

38. (Amended) Mutated *mspA* gene, *mspC* gene or *mspD* gene, [in particular] according to claim 36 [or 37], derived from one of the sequences 1, 6, or 8, in which the mutation is such that the chemical and thermal stability, as well as the channel-like structure of the protein is for all practical purposes that of MspA, MspC or MspD.

39. (Amended) Mutated *mspA* gene [according to claim 36 through 38], wherein the mutated gene is the *synmspA* gene according to sequence 4.

[redacted]

Methods for the Production of a Channel-forming Protein

The invention relates to a method for the production of a channel-forming protein, a channel-forming protein, a gene encoding such a protein and mutated *mspA*, *mspC* or *mspD* genes, a plasmid vector and an overexpression system.

The invention relates in general to the technical field of the production of nanostructures. To date, the best characterised nanostructures are the carbon nanochannels (Yakobson, B. I. und Smalley, R. E. Fullerene nanotubes: $C_{1,000,000}$ and beyond. *Am Sci* **85**, 324, 1997). It was shown that the electronic properties of carbon nanochannels could be controlled through their structural details. Carbon nanochannels are synthesised using different variants of CVD (chemical vapour deposition) methods (Fan, S., Chapline, M. G., Franklin, N. R., Tombler, T. W., Cassell, A. M. und Dai, H. Self-oriented regular arrays of carbon nanotubes and their field emission properties. *Science* **283**, 512-4,1999), which therefore is very sumptuous.

From Johnson, S. A., Ollivier, P. J. and Mallouk, T. E. „Ordered mesoporous polymers of tuneable pore size from colloidal silica templates." *Science* **283**, 963-965 (1999) a technique for creating organic nanochannels on the basis of a template is reported. With this process, nanochannels with a diameter from 5 to 35 nm can be produced.

Mycobacteria belong to a subgroup of Gram-positive bacteria, which contain mycolic acids and include the genera *Corynebacterium, Nocardia, Rhodococcus, Gordona, Tsukamurella, Dietzia.*

Trias, J. and Benz, R. „Permeability of the cell wall of *Mycobacterium smegmatis*." *Mol Microbiol* **14**, 283-290 (1994) describe channel-forming proteins, called porins, in the mycolic

acid layer of mycobacteria. Biochemical or molecular genetic data of these porins have not been published yet.

From Lichtinger, T., Burkovski, A., Niederweis, M., Kramer, R. and Benz, R. „Biochemical and biophysical characterization of the cell wall porin of *Corynebacterium glutamicum*: the channel is formed by a low molecular mass polypeptide." *Biochemistry* **37,** 15024-32 (1998) the technique is known to prepare porins from corynebacteria. This technique is relatively inefficient, though.

Mukhopadhyay, S., Basu, D. and Chakrabarti, P. „Characterization of a porin from *Mycobacterium smegmatis.*" *J Bacteriol* **179,** 6205-6207 (1997) describe the extraction of porins from *M. smegmatis* with a buffer containing 1 % Zwittergent by incubation at room temperature for one hour. The yields were poor and the porins were contaminated with many other proteins.

From Harth, G. et al., „High-level heterologous expression and secretion in rapidly growing nonpathogenic mycobacterium of four major *Mycobacterium tuberculosis* extracellular proteins considered to be leading vaccine candidates and drug targets." *Infection and Immunity* **65,** 2321-2328 (1997) it is known that a strong expression of *Mycobacteria*-specific proteins in *E. coli* seems to be not possible.

Senaratne, R.H. et al., „Expression of a Gene for a Porin-Like Protein of the OmpA Family from Mycobacterium t*uberculosis* H37Rv." *J Bacteriol* **180,** 3541-3547 (1998) describe the expression of a gene for a porin-like protein from *Mycobacterium tuberculosis* H37Rv in *E. coli*. The expression of the gene causes a discontinuance in the growth of the bacteria, evidently because the expressed protein is toxic for *E. coli*. A negligible amount of protein from *E. coli* could be isolated shortly before the dying of the cells.

It is an object of the invention, to provide an improved method for the production of a channel-forming protein.

This object is solved by the features of Claims 1, 26, 27, 28, 30, 32, 36, 37, 38, 40 and 41. Further suitable embodiments derive from the features of Claims 2 through 25, 29, 31, 33, 34, 35, 39, and, optionally, 37 and 38.

According to the invention, a method is provided for producing a channel-forming protein, found in Gram-positive bacteria, in which the channel-forming protein is produced through

a) heterologous overexpression or

b) purification from mycobacteria, wherein the extraction temperature is higher than 50°C.

When referring to channel-forming proteins, a protein is meant, which can form a water-filled channel or a water-filled channel-like structure. Such proteins occur naturally, especially in the cell walls of bacteria. They can form channel-like structures with diameter up to 3 nm or even larger. The length can be up to 10 nm or more. The channel-like structures or channels can be made up of many substructures, specifically 4 or 8 substructures.

This method according to the invention is much more efficient in comparison to the prior procedures, offers the possibility of a far-reaching automation of the chromatographic purification, and allows for a drastically increased yield.

The gram-positive bacterium can be a bacterium, which contains at least one mycolic acid. In the described According to one

embodiment, the bacterium is a mycobacterium, preferably *Mycobacterium smegmatis*.

The channel-forming protein can be a porin. Preferred is a porin, which is chemically stable in organic solution and/or thermally stable up to a temperature of 80°C, more preferably 100°C. Stable shall mean that the channel-like structure of the protein remains intact and essentially no denaturation of the protein occurs.

Preferred are the porins MspA, MspC, MspD, a fragment of these porins, a protein homologous to these porins or their fragments, or a protein of a sequence derived from these porins. MspA corresponds to the sequence of the amino acids 28 - 211 of sequence 3 (see below), MspC corresponds to sequence 7 and MspD corresponds to sequence 9. The homologous protein of said porins or their fragments exhibit a similar structure to that of said porin or their fragments. At least 20% of the amino acids are identical or homologous to the amino acids of these porins or fragments. An amino acid in a protein is homologous with another amino acid if it can be substituted with the other amino acid, without influencing the function or structure of the protein. A protein that has been deduced from a sequence of a porin can be missing a single or several amino acids when compared to the sequence, or contain other amino acids or amino acid analogues.

Said proteins are particularly suitable for the production of nanostructures because of their surprisingly high chemical and thermal stabilities.

A good yield will be obtained, if the heterologous overexpression is performed in *E. coli* or in mycobacteria. For overex-

pression, a gene encoding a channel-forming protein, preferably a porin, should be used. For overexpression, it is preferable to use an *mspA* gene according to sequence 1 (see below), an *mspC* gene according to sequence 6 or an *mspD* gene according to sequence 8. A mutant gene derived from the sequences 1, 6, or 8 can be used for overexpression, whereby the mutation is so that the chemical and thermal stability, as well as the channel-like structure, essentially correspond to that of MspA, MspC or MspD. The mutation can also be such that the codon usage of the *mspA*, *mspC* or *mspD* gene is adapted to that of highly expressed genes in *E. coli*. These codons are known from Nakamura, T. et al., „Two types of linkage between codon usage and gene-expression levels." *FEBS Lett.* **289**, 123-125 (1991).

A mutant *mspA*, *mspC* or *mspD* gene can also be used for overexpression, if the mutation essentially reduces the G+C content to less than 66%. The adaptation of the codon usage dramatically improves the overexpression of MspA, MspC and MspD in *E. coli*.

The yield of the channel-forming protein MspA can be further increased by a factor 10 to 20 through overexpression in *E. coli* compared to the method for preparation of the native protein described above.

It is useful to use the *synmspA* gene according to sequence 4 for overexpression. An overexpression vector for *E. coli*, in which the *synmspA* gene according to sequence 4 is inserted, can be used for this purpose. Suitable vectors are described by Hannig, G. and Makrides, S.C. in „Trends in Biotechnology", 1998, Vol. 16, pp54. The disclosure of this document is incorporated herein.

It has also been advantageously found to harvest the channel-forming proteins from the cell wall of the gram-positive bac-

teria by using non-ionic or zwitterionic detergents. The detergents can be selected from the following group: isotridecyl poly(ethylene glycolether)$_n$, alkyl glucosides, in particular octyl glucoside, alkyl maltosides, in particular dodecyl maltoside, alkyl thioglucosides, in particular octyl thioglucoside, octyl-polyethylenoxides and lauryl dimethylaminoxide. A twofold or higher critical micellar concentration (CMC) in a phosphate buffer (100 mM Na_2HPO_4/NaH_2PO_4, pH 6.5, 150 mM NaCl) is preferably used. The zwitterionic and non-ionic detergents very effectively dissolve the channel-forming protein MspA from the cell wall of *M. smegmatis*, resulting in a good yield.

It has further been shown as useful that the extraction temperature is between 80 and 110 °C, preferably between 90 and 100 °C and/or the extraction time is 5 - 120 min, preferably 25 - 35 min. Particularly preferred is the use of a buffer with an ionic strength of more than 50 mM NaCl or Na-phosphate.

In particular, performing the extraction at 100 °C and the use of a buffer with a high ionic strength or zwitterionic and non-ionic detergents will improve the method for extraction of porins from *Mycobacterium smegmatis*. In comparison with the prior procedures for purification of such proteins using organic solvents or their extraction at room temperature, it offers the following advantages:

aa) no use of organic solvents required
bb) minimal contamination with other proteins
cc) efficient extraction

It is also possible to purify MspA by dissolving it in dimethyl-sulfoxide at a temperature in the range from 50 - 110 °C; afterwards the solution is allowed to cool to room temperature, permitting filtration of the MspA precipitate.

Preferably, the channel-forming protein is precipitated, in particular using acetone, for purification. This procedure can result in the further concentration of MspA with respect to other non-precipitating proteins. It is also advantageous to purify the protein using an ion-exchange chromatography method, especially an anion-exchange chromatography method. Further purification can be achieved employing size-exclusion chromatography.

The renaturation of the channel-forming protein generated by means of heterologous overexpression, can be achieved by increasing the local concentration of the protein. The increase can be achieved using electrophoretic concentration, especially by means of a DC current, by precipitation or adsorption at a suitable surface (e.g. a membrane). Useful is a DC current of 50 V for 30 min.

Another aspect of the invention is a channel-forming protein from a gram-positive bacterium, produced according to the method of the invention.

The gram-positive bacterium can be a bacterium containing mycolic acids, whereby it is advantageous to use a mycobacterium, preferably *Mycobacterium smegmatis*.

It is especially advantageous that the channel-forming protein is a porin, which is chemically stable against organic solvents. The porin is essentially thermally stable up to a temperature of 80 °C, preferably up to 100 °C. This thermal stability is displayed by either MspA, MspC, MspD, a fragment of these porins, or a homologous porin to one of these fragments, or a porin which is derived from a sequence of the porins (MspA, MspC, MspD) or their fragments. The chemical and thermal stability, as well as the channel-forming structure of

such derived proteins, can in general correspond to the stabilities of the MspA, MspC, MspD proteins. It is further possible that additional proteins, which are not mentioned here, possess very similar properties and are therefore encompassed by the scope of the present invention.

The channel-forming proteins according to the present invention have the following advantages:

aaa) If the channel-forming proteins are found in the cell wall of *M. smegmatis*, they can be dissolved in organic solvents (e.g. $CHCl_3$/MeOH) without denaturation. The channel-forming property remains also in organic solvents.

bbb) They can be precipitated using acetone without denaturation.

ccc) They even survive being boiled in detergents (e.g. 10 min in 3% SDS) without denaturation.

This extreme stability of the inventive proteins against chemical and thermal denaturation makes it possible to use them in order to produce technically applicable nanostructures.

According to invention, a gene is furthermore claimed, which encodes a channel-forming protein according to the invention. This can be the *mspA* gene according to sequence 1, the *mspC* gene according to sequence 6 or the *mspD* gene according to sequence 8.

As an additional matter, a mutated *mspA* gene, *mspC* gene or *mspD* gene is provided, in which the codon usage of the aforesaid gene is practically the same as the codon usage of highly expressed *E. coli* genes. The mutation can be such that the G+C

content is reduced to less than 66%. The mutated gene can also be derived from one the sequences 1, 6, or 8 in such a way that the chemical and thermal stability, as well as the channel-like structure of the expressed protein, is essentially the same as that of MspA, MspC or MspD. Additional mutations that are not mentioned here are conceivable for the skilled artisan. Genes that lead to the formation of channel-like proteins according to the invention are herewith included in the scope of protection as claimed, e.g. a mutated *mspA* gene, in which the mutated gene is the *synmspA* gene according to sequence 4 (see below).

An additional object of the present invention is the plasmid vector pMN501 and an overexpression system, wherein *E. coli* contains said plasmid vector.

In the following examples of the invention are explained with reference to the figures. These show:

Fig. 1a-c the temperature dependent extraction of MspA from *M. smegmatis* as shown by gel electrophoresis,

Fig. 2 the purification of MspA from *M. smegmatis* as shown by gel electrophoresis,

Fig. 3 the purification of MspA from *E. coli* as shown by gel electrophoresis,

Fig. 4 the construction of plasmid vector pMN501,

Fig. 5 a scheme depicting an apparatus for renaturing monomeric MspA,

Fig. 6 renatured MspA as shown by gel electrophoresis and

Fig. 7a-c modifications of the channel-forming protein MspA as shown by electron microscopy.

The proteins were incubated at room temperature for 30 minutes in a sample buffer containing 40 mM tris(hydroxymethyl)aminomethane, pH 7.0, 3% sodium dodecyl sulfate, 8% glycerol, 0.1% Serva Blue G) in all gel electrophoretic experiments and then separated according to their sizes by gel electrophoresis.

The figures 1a-c show proteins extracted from *M. smegmatis* at different temperatures.

Fig. 1a. 10% denaturing polyacrylamide gel stained with Coomassie Blue. Lane M: molecular weight marker (200, 116.3, 97.4, 66.3, 55.4, 36.5, 31, 21.5 und 14.4 kDa). Lanes 1 through 8: 12 µl of each extract obtained at 30, 40, 50, 60, 70, 80, 90 and 100°C.

Fig. 1b shows an immunoblot analysis of an 8% denaturing polyacrylamide gel blotted onto a PVDF membrane. Proteins were visualized using an MspA antiserum and a chemoluminescence reaction (ECL detection system, AmershamPharmacia, Vienna, Austria). Lane M: molecular mass marker (97.4, 68, 46, 31, 20.1, 14.4 kDa kDa); lanes 1 through 3: 2 µL of extracts obtained at 30, 40 or 50 °C; lanes 4 through 8: 1 µL of extracts obtained at 60, 70, 80, 90 or 100 °C; (9) 1 ng MspA.

Fig. 1c shows a denaturing 8% polyacrylamide gel stained with silver. Lane M: molecular mass marker (200, 116.3, 97.4, 66.3, 55.4, 36.5, 31, 21.5, 14.4, 6 kDa kDa); lanes 1 and 2: 15 µL of extracts obtained at 30 and 40°C, respectively; lane 3: 10 µL of an extract obtained at 50°C, lanes 4 through 8: 4 µL of extracts obtained at 60, 70, 80, 90 or 100 °C; (9) 270 ng purified MspA.

Fig. 2 shows a denaturing 10% polyacrylamide gel stained with Coomassie Blue.

Lane M: molecular mass marker (200, 116.3, 97.4, 66.3, 55.4, 36.5, 31, 21.5, 14.4, 6 kDa kDa); Lane 1: 40 µg protein of an extract from *M. smegmatis* obtained using POP05 buffer (100 mM Na_2HPO_4/NaH_2PO_4, pH 6.5, 0.1 mM EDTA, 150 mM NaCl, 0.5 % octyl-polyethylenoxide (OPOE)). Lane 2: 40 µg protein of an extract after precipitation with acetone. Lane 3: 4 µg protein after anion-exchange chromatography and pooling of the fractions, which contained MspA. Lane 4: 4 µg protein of the pooled MspA fractions after precipitation with acetone. Lane 5: 4 µg protein after size-exclusion chromatography and pooling of the fractions, which contained MspA. The sequences of the *mspA* gene, of the *mspA* gene + promoter and the MspA protein with the putative signal sequence are shown as sequences 1 to 3 in the sequence protocol.

Fig. 3 shows the purification of the channel-forming protein MspA from *E. coli*. The proteins were separated according to their sizes in a 10% denaturing polyacrylamide gel. The gel was stained with Coomassie Blue. Lane 1: Lysate from *E. coli* BL21(DE3)/pMN501 before induction with IPTG. Lane 2: Lysate from *E. coli* BL21(DE3)/pMN501 after induction with IPTG. Lane 3: molecular mass marker (200, 116.3, 97.4, 66.3, 55.4, 36.5, 31, 21.5, 14.4, 6 kDa kDa). The samples were incubated for 30 minutes at 37 °C before loading on the gel.

Fig. 4. The construction of the vector pMN501 for overexpression of MspA in *E. coli* BL21(DE3) is schematically depicted. The meaning of the abbreviations is as follows:

lacI: gene encoding the lactose repressor

nptI: gene encoding the neomycine phosphotransferase. This gene confers resistance against kanamycin.
Ori: origin of replication
RBS: ribosomal binding site

Fig. 5 shows schematically an apparatus for renaturation of monomeric MspA. A dispensable pipette tip from polyethylene of 5 cm length was shortened by 2 mm at its lower end. This tip was filled with a solution of 1.7% agarose in TAE buffer. The lead of a pencil (brand: Eberhard Faber, 3H) was shortened to 5 cm. A tube from polypropylene without a lid was filled with 60 µl of a solution containing 5 µg denatured MspA. The pipette tip and the lead were put into the solution and connected as cathode and anode, respectively.

Fig. 6 shows the renaturation of denatured MspA. The proteins were separated in a 10% denaturing polyacrylamide gel as described by Schägger (Schägger, H. and von Jagow, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 166, 368-79 (1987)). The gel was stained with silver. Lane M: molecular mass marker (116, 97, 66, 55, 36.5, 31, 21.5, 14.4kDa). Lane 1: 800 ng denatured MspA. Lane 2: 800 ng MspA after the renaturation reaction. The samples were incubated for 30 minutes at 37 °C before loading on the gel.

Fig. 7a through 7c show electron microscopic pictures of modifications of the channel-forming protein MspA from *M. smegmatis*. The preparation of the sample was performed as follows:

One milliliter of a solution of MspA (c(MspA)= 17.2 x 10^{-9} mol/L, 100 mM Na_2HPO_4/NaH_2PO_4, pH 6.5, 150 mM NaCl, 0.10 g/L SDS) was dispersed by ultrasonication in a water bath at 24.5°C. The distance between the liquid and HOPG (carbon) sur-

faces (1,0 mm^2) was 5.0 cm. The dispersed liquid droplets were allowed to contact the HOPG surface for 20 seconds.

Fig. 7a shows isolated channel-forming proteins. Fig. 7b shows a ribbon-like structure, which exhibits large pores with a diameter of 12 nm. Fig. 7c shows two types of channels in the ribbon-like structure: Channels with a small diameter of about 2.4 nm and channels with a larger diameter of about 9 to 10 nm.

Example 1: Extraction of MspA from *M. smegmatis* at different temperatures

Ten milligrams of *M. smegmatis* mc^2155 cells (wet weight) were washed with PBS (100 mM sodium phosphate, pH 7,0, 150 mM NaCl, 0.1 mM EDTA) and resuspended in 150 µl PG05 buffer (0.5% isotridecylpolyethylenglycolether, 100 mM Na_2HPO_4/NaH_2PO_4, 0.1 mM EDTA, 150 mM NaCl, pH 6.5). The resuspended cells were incubated for 30 minutes at 30, 40, 50, 60, 70, 80, 90 or 100°C. The samples were cooled on ice for 10 minutes and centrifuged for 10 minutes at 4°C. The volume of the supernatant was reduced from 120 µl to 10 µl by evaporation. The proteins were separated according to their sizes by gel electrophoresis as it is shown in the Figures 1a-c. The fraction of MspA compared to the total protein in the extract increases significantly at temperatures above 50°C. At those temperatures, only minor amounts of other proteins are extracted.

Example 2: Purification of MspA from *M. smegmatis*

Ten grams *M. smegmatis* cells were washed with PBS, resuspended in 35 mL POP05 and boiled under stirring for 30 minutes in a water bath. The cell suspension was cooled on ice for 10 minutes, and centrifuged at 4°C for 15 minutes at 27000 g. Forty-two milliliters of the supernatant were gently mixed with an

equal volume of ice-cold acetone. This mixture was kept on ice for one hour and centrifuged at 4°C for 15 minutes at 8000 g. The precipitated protein was dissolved in 10 mL 25mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethane sulfonic acid (HEPES), pH 7.5, 10 mM NaCl, 0.5% OPOE (AOP05) and loaded on an anion exchange column "POROS 20HQ" with a volume of 1.7 mL (Perseptive Biosystems, Cambridge, USA). After washing the column with 14 mL AOP05, bound proteins were eluted with a gradient from 100% AOP05 to 100% BOP05 (25 mM HEPES, pH 7.5, 2 M NaCl, 0.5% OPOE) over 34 mL. Ninety fractions of 1 mL were collected and analysed by gel electrophoresis. Four fractions with the highest amount of MspA were pooled and the protein was precipitated with acetone as described above. The pellet was dissolved in 600 µL AOP05, incubated on ice and centrifuged at 4 °C for 5 minutes to remove insoluble material. The protein solution was loaded on a gel filtration column "Superdex G200" with a volume of 24 mL (Pharmacia, Freiburg, Germany). Proteins were eluted with 48 mL of AOP05 at a flow rate of 0.2 mL/min. Fifty fractions of 1 mL were collected and analyzed using denaturing polyacrylamide gels which were stained with silver. Fractions containing apparently pure MspA were pooled. The purification steps are shown in Fig. 2. The yield was 700 µg. 1 µg of this sample Probe did not show any contamination with other proteins in a silver-stained denaturing polyacrylamide gel (data not shown). Thus, MspA was purified to apparent homogeneity.

Example 3: Strategy for purification of the channel-forming protein MspA from *E. coli*

To further increase the yield of MspA, an overexpression of the *mspA* gene in *E. coli* is suggested. The *mspA* gene, which encodes the channel-forming protein MspA from *M. smegmatis*. The T7 expression system is chosen for overexpression of the *mspA* gene.

The *mspA* gene was amplified from the plasmid pPOR6 by PCR. All codons of the native mspA sequence, which occur rarely in highly expressed genes in *E. coli*, were exchanged. All mutations are listed in sequence 4 of the sequence protocol (see below). This gene was synthesized by assembling of oligonucleotides as described by Stemmer (Stemmer, W. P., Crameri, A., Ha, K. D., Brennan, T. M. and Heyneker, H. L. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. *Gene* **164**, 49-53 (1995)) and was called *synmspA*. The *synmspA* gene replaced the *mspA* gene in the vector pMN500, whose use did not lead to detectable amounts of MspA in *E. coli*, to give the vector pMN501 (Fig. 4). The vector pMN501 gave rise to a strong expression of the MspA monomer (20 kDa) in *E. coli* BL21(DE3) after induction with IPTG. This protein is called recombinant MspA (rMspA) and possesses the sequence 5 of the sequence protocol (see below).

Example 4: Procedure for purification of the channel-forming protein MspA from *E. coli*

One litre LB medium containing 30 µg/mL kanamycin is inoculated with mit *E. coli* BL21(DE3)/pMN501 and the culture was grown to an OD_{600} of 0.6 at 37 °C. Then, the cells are induced with 1 mM IPTG and are incubated at 37 °C for further six hours, until the culture reaches an OD_{600} of 2.2. The cells are harvested by centrifugation, resuspended in 40 mL A-Puffer (25 mM Hepes, pH 7,5, 10 mM NaCl) and lysed by boiling in water for 10 min. The cell lysate is kept on ice for 10 min and cell debris and insoluble proteins are precipitated by centrifugation at 10000 g for 10 min. The supernatant is fractionated using anion exchange chromatography (POROS HQ20, Perseptive Biosystems, Cambridge, USA) and a linear gradient from 10 mM to 2 M sodium chloride. Monomeric MspA elutes at 350 mM NaCl. The fractions containing MspA are pooled. Size-exclusion chromatography (Superdex G200, Pharmacia, Freiburg, Germany)

is used to purify MspA from proteins with a larger molecular weight. The yield is 10 mg MspA with a purity exceeding 95% (data not shown).

Example 5: Electrochemical assembly of the channel-forming protein MspA

Overexpression of MspA in *E. coli* easily allows to produce MspA with a good yield. However, a large fraction of the purified protein is inactive. Renaturation of MspA into its active form can be achieved by using the following protocol:
Renaturation can take place in an apparatus specially designed for this purpose (Fig. 5). The renaturation reaction is performed with 5 μg monomeric MspA in the aforementioned apparatus by applying a voltage of 50 V for 30 min. Then, the sign of the applied voltage is reversed for five seconds, to remove porin adsorbed at the surface of the lead. The protein is analysed in a denaturing denaturing polyacrylamide gel after the renaturation reaction (Fig. 6). This gel shows that a large fraction of the protein is assembled to oligomers. It is demonstrated by reconstitution in lipid bilayer experiments, that this assembled MspA has a high channel-forming activity. This experiment demonstrates that renaturation of monomeric MspA is possible using small DC voltages. This renaturation reaction is very easy to perform and is an important component of the purification of functional MspA from overexpressing *E. coli*.

List of sequences:

1. *mspA* gene, translated
2. *mspA* gene + promoter, translated
3. MspA protein with putative signal sequence
4. *synmspA* gene, translated
5. rMspA protein
6. *mspC* gene
7. MspC protein

8. *mspD* gene

9. *mspD* protein

SEQUENCE PROTOCOL

<110> Niederweis Dr., Michael

Bossmann Dr., Stefan

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Leu Ser Leu Val Asp Gly Gln Asp Arg Thr Leu Thr Val Gln Gln Trp
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 65                  70                  75                  80

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Asp Thr Phe Leu Asn Gly Val Phe Pro Leu Asp Arg Asn Arg Leu Thr
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Gly Ala Asp Glu Phe Glu Gly Thr Leu Glu Leu Gly Tyr Gln Ile Gly
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Phe Pro Trp Ser Leu Gly Val Gly Ile Asn Phe Ser Tyr Thr Thr Pro
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Asn Ile Leu Ile Asp Asp Gly Asp Ile Thr Ala Pro Pro Phe Gly Leu
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Asn Ser Val Ile Thr Pro Asn Leu Phe Pro Gly Val Ser Ile Ser Ala
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Asp Leu Gly Asn Gly Pro Gly Ile Gln Glu Val Ala Thr Phe Ser Val
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Asp Val Ser Gly Ala Glu Gly Gly Val Ala Val Ser Asn Ala His Gly
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Thr Val Thr Gly Ala Ala Gly Gly Val Leu Leu Arg Pro Phe Ala Arg
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145                 150                 155                 160

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Ala Pro Pro Phe Gly Leu Asn Ser Val Ile Thr Pro Asn Leu Phe Pro
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Val Ser Asn Ala His Gly Thr Val Thr Gly Ala Ala Gly Gly Val Leu
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Pro Phe Gly Leu Asp Thr Ile Ile Thr Pro Asn Leu Phe Pro Gly Val
            100                 105                 110

Ser Ile Ser Ala Asp Leu Gly Asn Gly Pro Gly Ile Gln Glu Val Ala
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Thr Phe Ser Val Asp Val Lys Gly Ala Lys Gly Ala Val Ala Val Ser
    130                 135                 140

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Pro Phe Ala Arg Leu Ile Ala Ser Thr Gly Asp Ser Val Thr Thr Tyr
                165                 170                 175

Gly Glu Pro Trp Asn Met Asn
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```

Patent Claims

1. A Method for producing a channel-forming protein, found in gram-positive bacteria, wherein the channel-forming protein is obtained by

a) heterologous overexpression or

b) purification from mycobacteria, whereby the extraction temperature is higher than 50°C.

2. A Method according to one of the aforementioned claims, wherein the gram-positive bacterium is one that contains at least one mycolic acid.

3. A Method according to one of the aforementioned claims, wherein the bacterium is a mycobacterium, preferably *Mycobacterium smegmatis*.

4. A Method according to one of the aforementioned claims, wherein the channel forming protein is a porin.

5. A Method according to one of the aforementioned claims, wherein the porin is essentially chemically stable against organic solvents.

6. A Method according to claim 4, wherein the porin is essentially thermally stable up to a temperature of 80°C, preferably 100°C.

7. A Method according to one of the aforementioned claims, wherein the porin is the porin MspA, MspC, MspD, a fragment of one of these porins, a homologous protein from one of these porins or their fragments, or a protein taken from a sequence of one of these porins.

8. A Method according to one of the aforementioned claims, wherein the heterologous overexpression is realized in *E. coli* or mycobacteria .

9. A Method according to one of the aforementioned claims, wherein a gene encoding a channel-forming protein, preferably a porin, is used for the overexpression.

10. A Method according to one of the aforementioned claims, wherein an *mspA* gene according to sequence 1, an *mspC* gene according to sequence 6, or an *mspD* gene according to sequence 8 is used for overexpression.

11. A Method according to one of the aforementioned claims, wherein a mutant gene derived from the sequences 1, 6, or 8 is used for overexpression, in which the mutation is essentially so that the chemical and thermal stability, as well as the channel-like structure, correspond essentially with that of MspA, MspC or MspD.

12. A Method according to one of the aforementioned claims, wherein the mutation is essentially so that the codon usage of the *mspA*, *mspC* or *mspD* gene is adapted to that of highly expressed genes in *E. coli*.

13. A Method according to one of the aforementioned claims, wherein a mutated *mspA*-, *mspC*- or *mspD* gene is used for overexpression where the mutation is essentially so that the G+C content is reduced to less than 66%.

14. A Method according to one of the aforementioned claims, wherein the *synmspA* gene according to sequence 4, is used for overexpression.

15. A Method according to one of the aforementioned claims, wherein a suitable vector for overexpression in *E. coli*, containing the *synmspA* gene according to sequence 4, is used.

16. A Method according to one of the aforementioned claims, wherein the channel-forming proteins are produced from the cell wall from gram-positive bacteria using non-ionic or zwitterionic detergents.

17. A Method according to one of the aforementioned claims, wherein the detergents used come from the following list: isotridecylpoly(ethyleneglycolether)$_n$, alkylglucosides, especially octylglucoside, alkylmaltoside, especially dodecylmaltoside, alkylthioglucosides, especially octylthioglucoside, octyl-polyethylenoxide and lauryldimethylaminoxide (??).

18. A Method according to one of the aforementioned claims, wherein the extraction temperature is between 80 and 110°C, preferably between 90 and 100°C.

19. A Method according to one of the aforementioned claims, wherein the extraction time is 5 - 120 min, preferably 25 - 35 min.

20. A Method according to one of the aforementioned claims, wherein a buffer with an ionic strength above 50 mM NaCl or Na-phosphate is used.

21. A Method according to one of the aforementioned claims, wherein the channel-forming protein is purified by precipitation, particularly using acetone.

22. A Method according to one of the aforementioned claims, wherein the channel-forming protein is purified using ion-

exchange chromatography, particularly an anion-exchange chromatography.

23. A Method according to one of the aforementioned claims, wherein the channel-forming protein is purified using size-exclusion chromatography.

24. A Method according to one of the aforementioned claims, wherein one of the aforementioned claims, wherein the channel-forming protein, produced through heterologous overexpression by raising the local concentration, is renatured.

25. A Method according to claim 24, wherein raising of the local concentration is realized by electrophoretic enrichment, especially by means of a DC current, by precipitation or adsorption at a suitable surface, especially at a membrane.

26. Channel-forming protein from a gram-positive bacterium, produced according to a method according to one of the aforementioned claims.

27. Channel-forming protein from a gram-positive bacterium, wherein the channel-forming protein is a porin that is essentially chemically stable against organic solvents.

28. Channel-forming protein from a gram-positive bacterium, wherein the channel forming protein is a porin essentially stable up to a temperature of 80°C.

29. Channel-forming protein according to claim 28, wherein the channel-forming protein is a porin that is essentially thermally stable up to a temperature of 100°C.

30. Channel-forming protein from a gram-positive bacterium, wherein the channel-forming protein is the porin MspA, MspC,

MspD, a fragment of these porins, a protein homologous to these porins or their fragments, or a protein derived from a sequence of these porins.

31. Channel-forming protein according to claim 30, wherein the chemical and thermal stability, as well as the channel-like structure of the deduced protein, is essentially that of the proteins MspA, MspC oder MspD.

32. Gene, encoding a channel forming protein according to one of the claims 26 - 31.

33. Gene according to claim 32, wherein the gene is the *mspA* gene according to sequence 1.

34. Gene according to claim 32, wherein the gene is the *mspC* gene according to sequence 6.

35. Gene according to claim 32, wherein the gene is the *mspD* gene according to sequence 8.

36. Mutated *mspA* gene, *mspC* gene or *mspD* gene, wherein the mutation is essentially such that the codon usage of the *mspA*, *mspC* or *mspD* gene is adapted to that of highly expressed genes in *E. coli*.

37. Mutated *mspA* gene, *mspC* gene or *mspD* gene, in particular according to claim 36, in which the mutation essentially consists of reducing the G+C content to less than 66%.

38. Mutated *mspA* gene, *mspC* gene or *mspD* gene, in particular according to claim 36 or 37, derived from one of the sequences 1, 6, or 8, in which the mutation is such that the chemical and thermal stability, as well as the channel-like structure

of the protein is for all practical purposes that of MspA, MspC or MspD.

39. Mutated *mspA* gene according to claim 36 through 38, wherein the mutated gene is the *synmspA* gene according to sequence 4.

40. Plasmid vector pMN501.

41. Overexpression system, in which *E. coli* contains the plasmid vector pMN501.

Abstract

The present application relates to a method for producing a channel-forming protein, found in gram-positive bacterium, wherein the channel-forming protein is prepared using

a) heterologous overexpression or

b) purification from mycobacteria, wherein the extraction temperature is higher than 50°C.

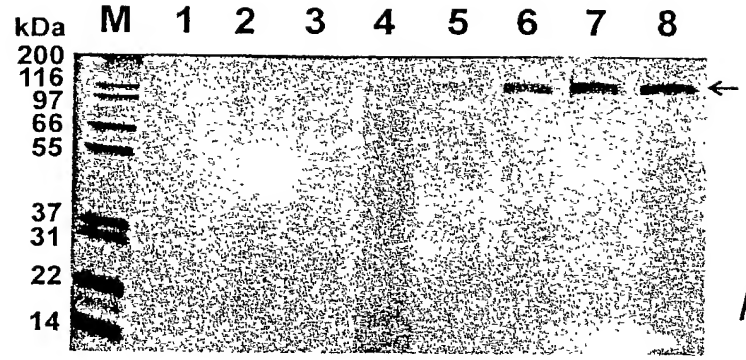

Fig. 1a

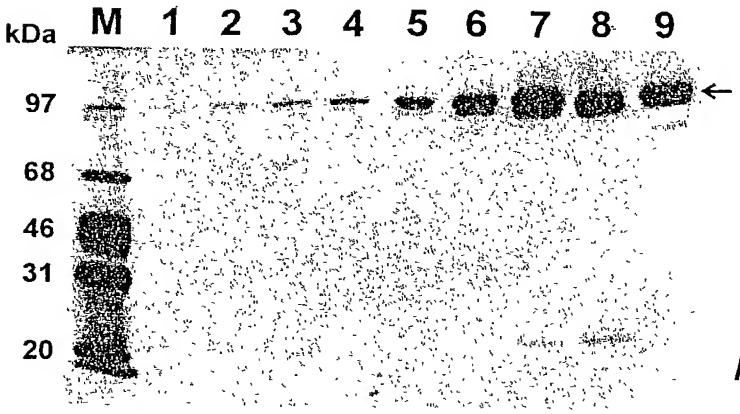

Fig. 1b

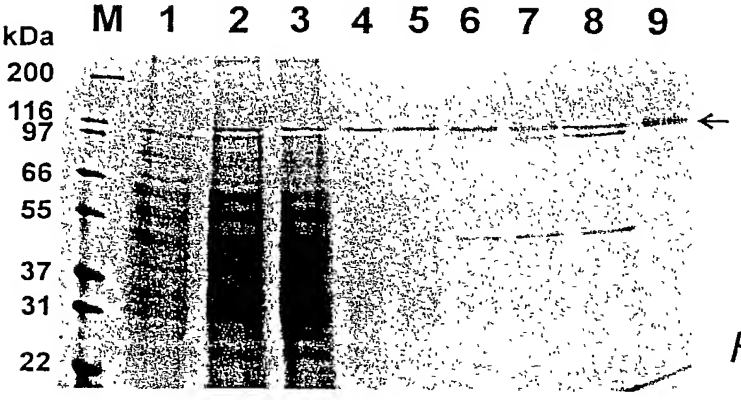

Fig. 1c

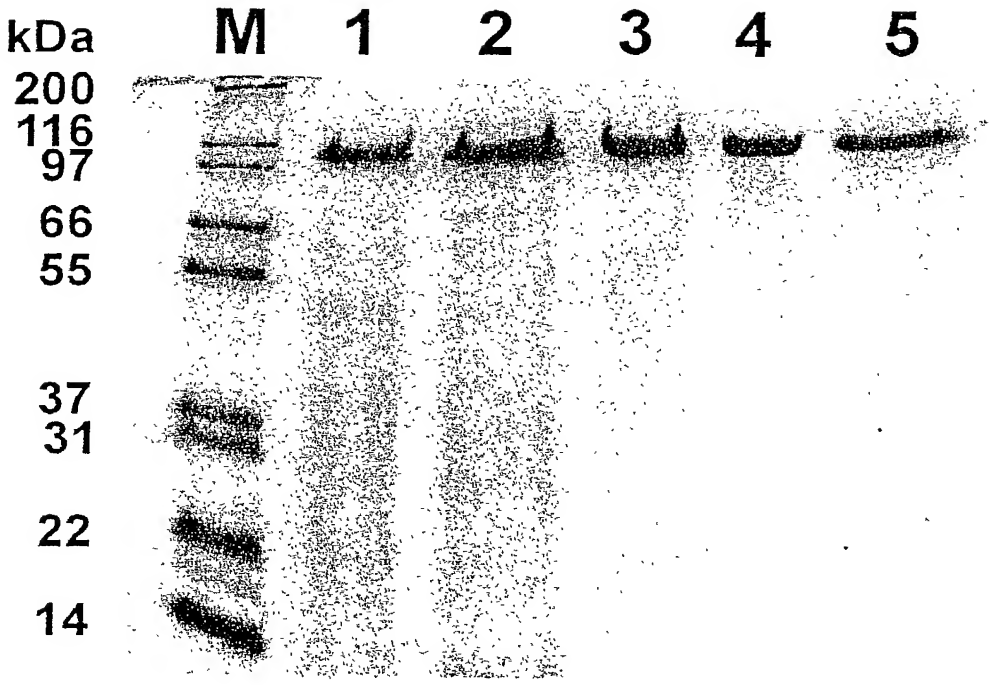

Fig. 2

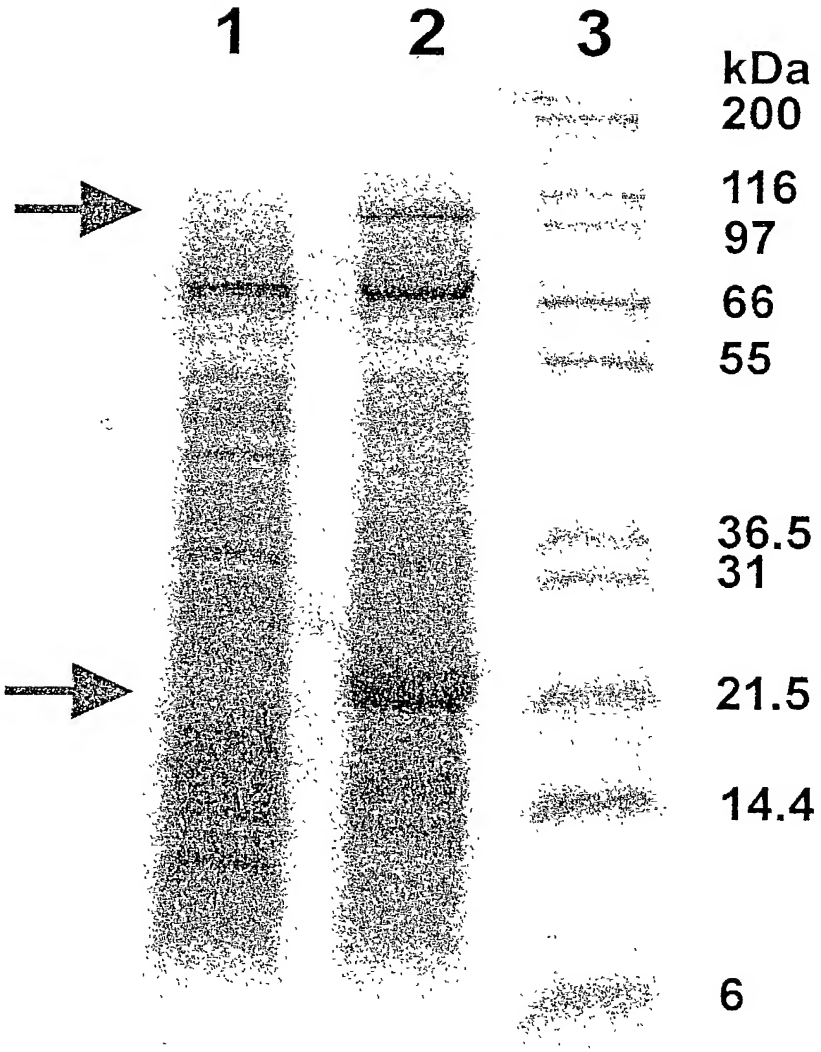

Fig. 3

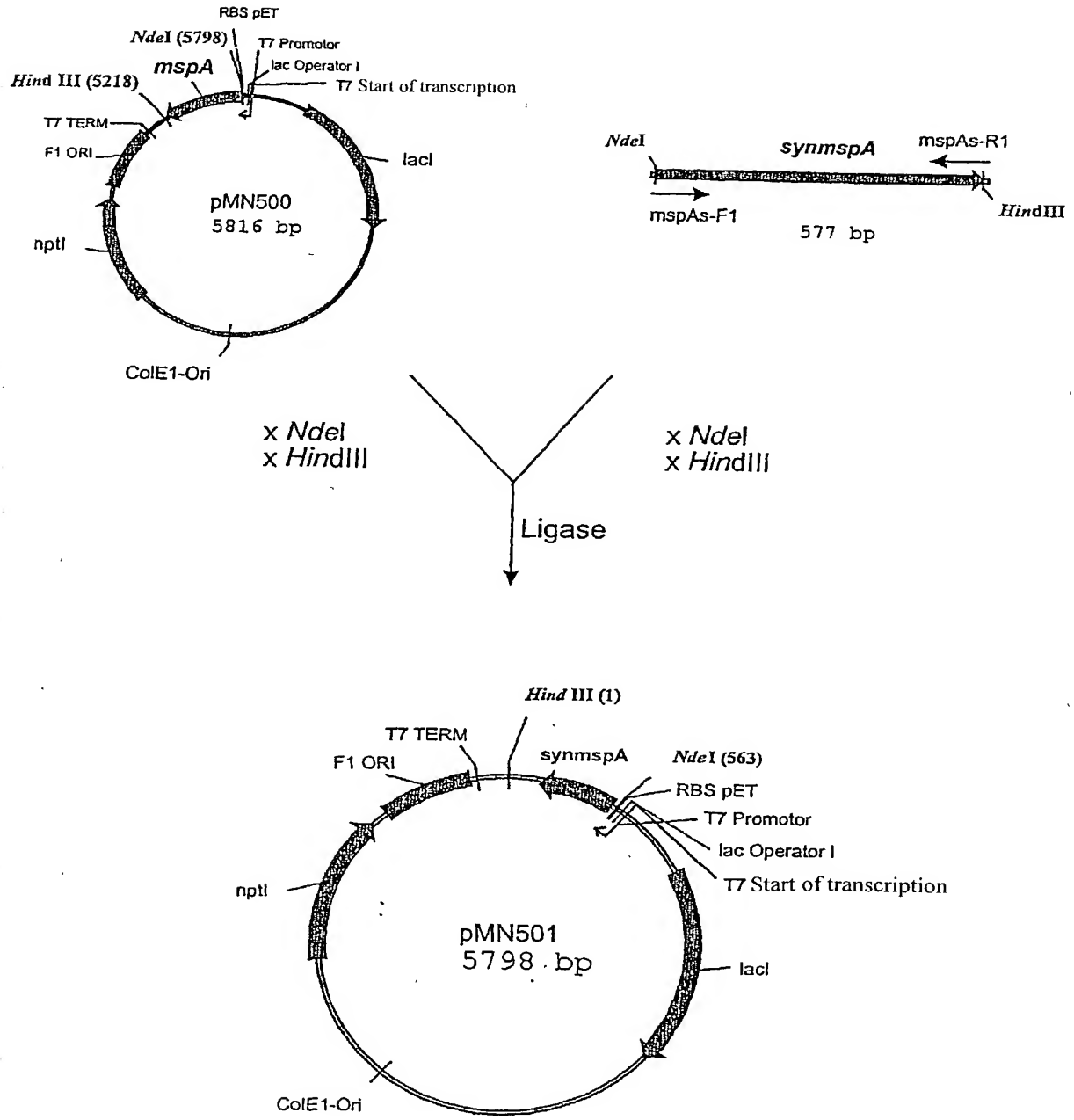
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Fig. 4

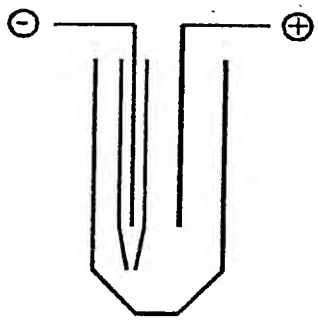

Fig. 5

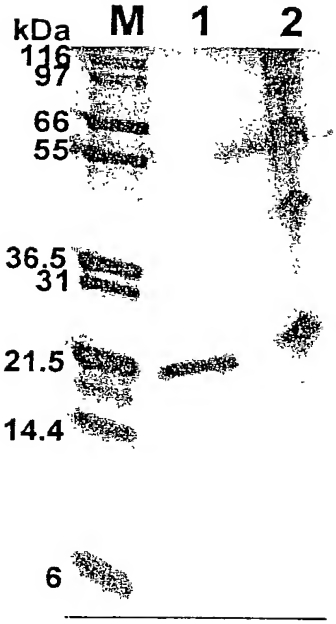

Fig. 6

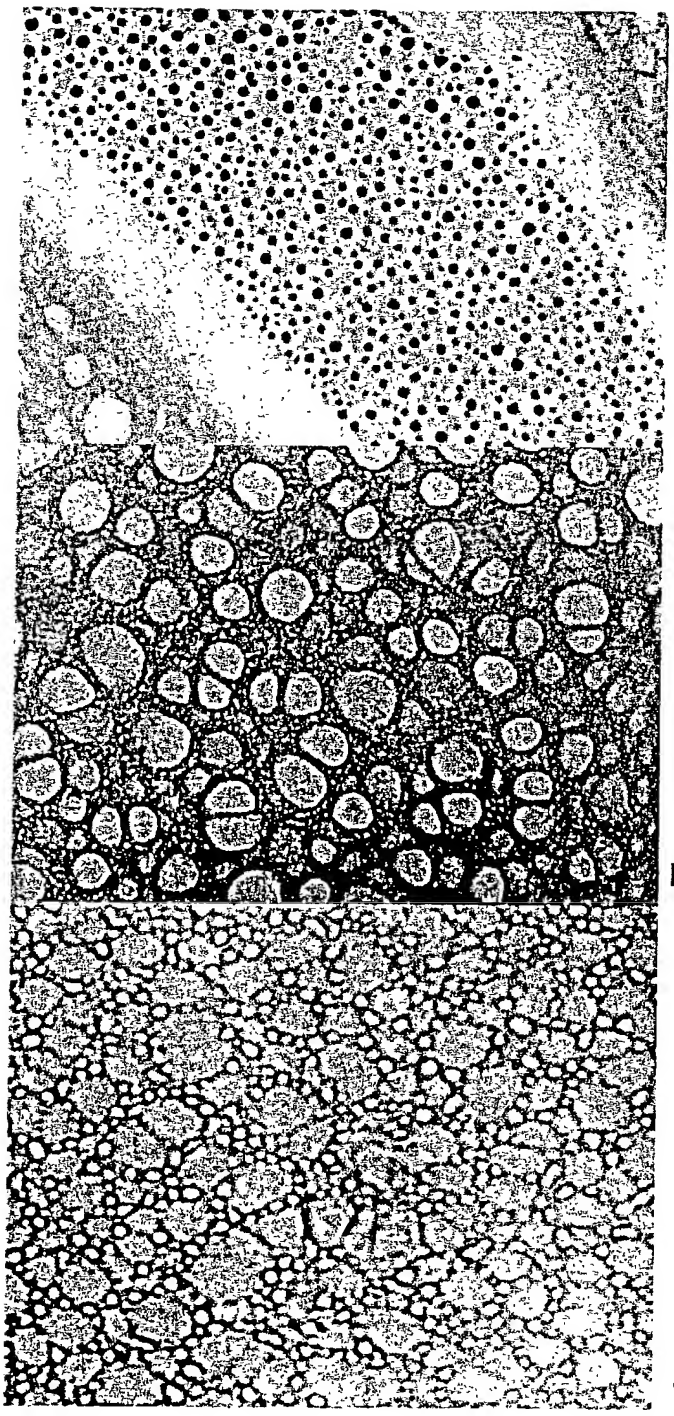

Fig. 7a

Fig. 7b

Fig. 7c

Attorney Docket No. 81839

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHODS FOR THE PRODUCTION OF A CHANNEL-FORMING PROTEIN, the specification of which: (check one)

[ ] is attached hereto.
[X] was filed as PCT International Application No. PCT/DE00/02924 on August 28, 2000.
[ ] was filed on
and assigned Serial No.:
and was amended on

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application on which priority is claimed:

Prior Foreign/PCT Application(s)			Priority Claimed
199 41 416.5	Germany	31 August 1999	[X] yes [ ] no
(number)	(country)	(day/month/year filed)	
199 43 520.0	Germany	11 September 1999	[X] yes [ ] no
(number)	(country)	(day/month/year filed)	

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

PROVISIONAL APPLICATION NUMBER	FILING DATE:
_____	_____
_____	_____
_____	_____
_____	_____

81839

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or under Title 35, United States Code, § 365(c) of any PCT international application(s) designating the United States listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations 1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(application number)	(filing date)	(Status - patented, pending, abandoned)
(application number)	(filing date)	(Status - patented, pending, abandoned)

I hereby appoint the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Irving M. Kriegsman, Esq., Reg. No. 22,733; Edward M. Kriegsman, Esq., Reg. No. 33,529; and Daniel S. Kriegsman, Esq., Reg. No. 40,057.

Please send all correspondence to:

KRIEGSMAN & KRIEGSMAN
665 Franklin Street
Framingham, MA 01702
(508) 879-3500

I hereby declare that all statements made herein of my own knowledge are true and that any statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of Inventor (given name, family name): MICHAEL NIEDERWEIS
Residence: KILLINGER STRASSE 108, 91056 ERLANGEN, GERMANY
Post Office Address: KILLINGER STRASSE 108, 91056 ERLANGEN, GERMANY
Citizenship: Germany

Inventor's signature:_____ Date:_____

Full Name of Inventor (given name, family name): STEFAN BOSSMANN
Residence: HAID- UND NEUSTRASSE 6, 76131 KARLSRUHE, GERMANY
Post Office Address: HAID- UND NEUSTRASSE 6, 76131 KARLSRUHE, GERMANY
Citizenship: Germany

Inventor's signature:_____ Date: 02/20/2002

81839

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or under Title 35, United States Code, § 365(c) of any PCT international application(s) designating the United States listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations 1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

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1-00

Full Name of Inventor (given name, family name): MICHAEL NIEDERWEIS
Residence: KILLINGER STRASSE 108, 91056 ERLANGEN, GERMANY DEX
Post Office Address: KILLINGER STRASSE 108, 91056 ERLANGEN, GERMANY
Citizenship: Germany

Inventor's signature: M. Niederweis Date: 02/02/19

2-00

Full Name of Inventor (given name, family name): STEFAN BOSSMANN
Residence: HAID- UND NEUSTRASSE 6, 76131 KARLSRUHE, GERMANY DEX
Post Office Address: HAID- UND NEUSTRASSE 6, 76131 KARLSRUHE, GERMANY
Citizenship: Germany

Inventor's signature:_____ Date:_____